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Characterization of Hepatitis E Virus (HEV) From Algeria and Chad by Partial Genome Sequence

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The purpose of this study was to analyze partial nucleotide sequences and derived peptide sequences of hepatitis E virus (HEV) from two outbreaks of hepatitis E in Africa (Chad 1983–1984; Algeria 1978–1980). A portion of ORF3 and the major portion of ORF2 were amplified by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The PCR products were sequenced directly or after cloning into the pCRII vector. Sequences were then compared to the corresponding regions of reported full length HEV sequences. In the ORF2 and ORF3 regions, the homology between the Algerian and the Chad isolates at the nucleic acid level was 92 and 95%, respectively. At the peptide level the homology was 98% in both regions. In these regions, both strains are more related to Asian strains at the nucleic acid level (89 to 95%) and at the amino acid level (95 to 100%) than to the Mexico strain. At the peptide level the differences are less apparent. Both African isolates have amino acid changes in common with some reference strains although the Chad isolate has three unique changes. These African strains of HEV, based on the ORF2 and ORF3 phylogenetic trees, appear to be a distinct phylogenetic group, separate from the Mexican and Asian strains. ***J. Med. Virol. 53:340–347, 1997.***

KEY WORDS: ORF2, ORF3 overlap; phylogeny; genomic variation; polymerase chain reaction

INTRODUCTION

Outbreaks of enterically-transmitted non-A, non-B (ET-NANB) hepatitis occur worldwide, including the former Soviet States, Asia, Africa, and Mexico [for reviews see Purcell and Ticehurst, 1988; Bradley, 1992; Mushahwar et al., 1993; Ticehurst, 1995; Purcell, 1996]. Direct proof that hepatitis E virus (HEV) was a cause of non-A, non-B hepatitis was shown by the enteral inoculation of HEV-containing feces in humans [Balayan et al., 1983; Chauhan et al., 1993] and primates [Andjaparidze et al., 1986; Gupta et al., 1990], resulting in disease, recovery of the agent, and seroconversion.

HEV is a 27–34 nm diameter, nonenveloped virus with physicochemical properties similar to caliciviruses. The HEV genome consists of a positive-sense, single-strand, polyadenylated RNA molecule of approximately 7.5 kb which encodes three open reading frames (ORF). ORF1 at the 5′ end of the genome encodes the nonstructural proteins. ORF2 encodes the putative viral structural protein. The function of the protein encoded by ORF3 is unknown.

The genome of HEV isolated in Pakistan, Sar-55 [Iqbal et al., 1989; Ticehurst et al., 1992; Tsarev et al., 1992], strains from Burma [Tam et al., 1991] and Myanmar [Aye et al., 1993], several strains from China [Huang et al., 1992; Bi et al., 1993; Yin et al., 1994; Uchida, unpublished data], a strain from India [Donati et al., 1996], and a strain from Mexico [Huang et al., 1992], are completely sequenced. Nucleic acid sequences of HEV from Asia are similar but as a group are different from the sequence of the strain isolated from Mexico. HEV has been implicated in several hepa-

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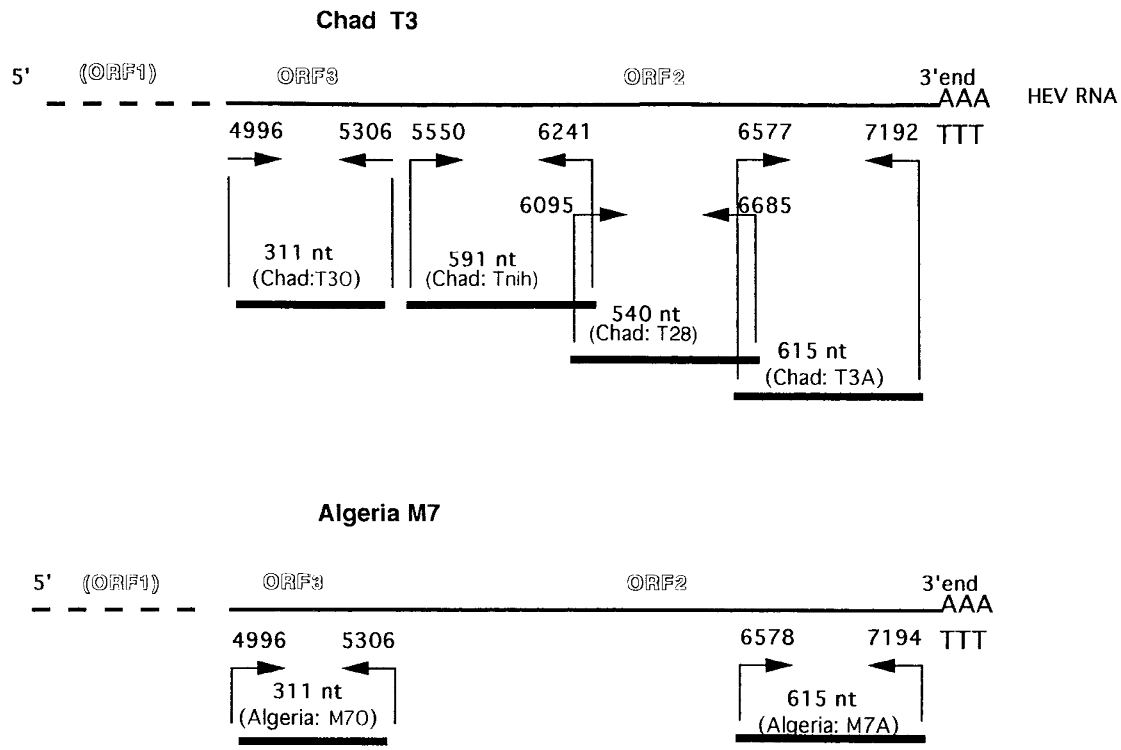

Fig. 1. PCR products obtained for Algeria (M7) and Chad (T3) isolates.

titis outbreaks in Africa [Belabbes et al., 1985; Molinie et al., 1986; Tsega et al., 1991; Coursaget et al., 1993b; Mushahwar et al., 1993; van Cuyck-Gandre et al., 1996], however no isolates from Africa have been sequenced.

Recently, using synthetic peptides [Yarbough et al., 1991; Kaur et al., 1992; Coursaget et al., 1993a,b; Khudyakov et al., 1993, 1994] and recombinant proteins [Dawson et al., 1992; Tsarev et al., 1993; Yarbough et al., 1996], immunodominant regions and a type-common specific epitope have been identified in the C-terminal region of the proteins encoded by ORF2 and ORF3. Strain variations in the epitope regions of the ORF2 and ORF3 could influence the serologic diagnosis of hepatitis E and the development of vaccines. Therefore, the analysis of the sequence diversity of HEV from multiple geographic origins is important.

We report the partial sequence of HEV isolated from two hepatitis outbreaks in North Africa. One outbreak occured in residents of Mostaganem, Algeria, in 1978–1980 (Coursaget et al., 1993b). The second occured in French soldiers stationed in Chad in 1983–1984 [Molinie et al., 1986]. HEV was identified as the causative agent of hepatitis by serology [Coursaget et al., 1993b] and confirmed by PCR amplification of HEV genome from stools of some patients [van Cuyck-Gandre et al., 1996]. The purpose of the current study was to analyze partial nucleotide and derived amino acid sequences of HEV from each outbreak and to compare them to previously reported sequences of other strains.

MATERIALS AND METHODS

HEV was detected in the feces of two hepatitis patients from the outbreaks: patient M7 from Algeria, 1980 (Mostaganem), and patient T3 from Chad, 1984 (N'Djamena). To describe the HEV analysis, terms must be defined. An isolate is defined as HEV contained in a specific patient specimen. A strain is an isolate which has been entirely sequenced and sufficiently characterized to serve as a reference. All nucleotide numbering refers to the Burma sequence [Tam et al., 1991].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For analysis of the ORF2 region, HEV was isolated from suspensions of feces (10% wt/vol in Phosphate Buffered Saline pH 7.9; PBS) by the affinity capture method described previously [Miele et al., unpublished data; van Cuyck-Gandre et al., 1996]. Serum from a Pakistani patient with acute hepatitis E was diluted to 1:1,000 to bind HEV in the fecal suspension. Complexes were then captured with anti-IgG (1:10,000 dilution; Tago, Inc., Burlingame, CA) coated on the walls of PCR tubes (Perkin-Elmer, Oak Brook, IL). Segments of the genome were amplified by RT-PCR using poly T primers and other PCR primers derived from the ORF2 and ORF3 of the Burma sequence [Tam et al., 1991] (Fig. 1). For analysis of the ORF3 region, RNA was extracted directly with TRIZol (GIBCO BRL/Life Tech-

nologies, SARL, Gaithersburg, MD) before amplification by nested RT-PCR.

The oligonucleotide primers were synthesized (Applied Biosystem 394, Foster City, CA) or purchased (Promega, Madison, WI). The reverse transcription was performed in 20 μl total volume, using 75 Units AMV-RT (Seikagaku America, Inc., Ijamsville, MD), 50 pmole antisense primer, 2.5 mM DTT, 10 mM dNTPs, and 1 × PCR buffer and incubated for 60 min at 42°C. Ten μl from the reverse transcription mix were used to perform a single round PCR amplification. The 100 μl PCR reaction final volume contained 50 pmoles each of sense and antisense primers, 1 × PCR buffer (final concentration), dNTPs 10 mM each and Taq polymerase 0.25 Unit (Perkin-Elmer). The PCR procedure consisted of incubation for 1 min at 95°C followed by 34 cycles each consisting of 1 min at 95°C, 1 min at 55°C, then 4 min at 72°C, followed by 1 cycle consisting of 1 min at 95°C, 1 min at 55°C, then a final incubation for 10 min at 72°C. The reaction mix was held at 4°C. The ORF3 region was amplified with nested PCR. The reaction for the ORF3 region was modified by adding glycerol to 5% of the final volume of the reaction mixture. For the nested amplification 5 μl of the first round product was transfered to the new reaction mixture and amplified under the same conditions as the first round. The PCR products were separated by electrophoresis in 1% or 2% agarose gel and detected by staining with ethidium bromide.

Cloning

The PCR products were purified by extracting DNA from agarose gel slices with phenol, then precipitating the DNA with 2-propanol. The DNA pellet was resuspended in water. The PCR products were inserted in the pCRII vector (TA Cloning kit, Invitrogen Inc., La Jolla, CA). Recombinant plasmids were transformed into $CaCl_2$ competent *E. coli* and kanamycin resistant colonies were selected. Each patient's identity, M7 or T3, was given to the first PCR product cloned. Further products were differentiated by numbers and/or letters. Sequence analysis was done on three clones from each recombinant plasmid.

Sequencing

DNA sequences were generated by a PRISM 377 automated DNA sequencer with PRISM dye Terminator Kits (Perkin-Elmer). The Dye terminator kit reactions were based on oligonucleotide primers synthesized from the Burma sequence [Tam et al., 1991], the sequence of the pCRII vector (Reverse primer sequence: 5′-AAC AGC TAT GAC CAT G-3′ and Forward primer sequence: 5′-GTA AAA CGA CGG CCA GT-3′), or sequences obtained from other portions of the HEV isolate.

Computer Analysis

The computer analysis of the sequencing reactions was performed using the Sequencer 3.0 program (Gene Codes Corp., Ann Arbor, MI). A consensus sequence was created from the sequence of three independent clones which were obtained from the same PCR product, or from direct sequencing of the PCR products. The nucleic acid sequences and the amino acid deduced sequences were compared with full length genome sequences: the China A [Aye et al., 1992] (GenBank accession #D11092), China B [Bi et al., 1993; Tam et al., 1991) (GenBank accession #M94177; #L08816), China C [Yin et al., 1994] (GenBank accession #L25595, #D10330), Pakistan [Tsarev et al., 1993] (GenBank accession #M80581), China D [Uchida, unpublished data] (GenBank accession #D11093), India [Donati et al., 1996] (GenBank accession #X98292), Burma [Tam et al., 1991] (GenBank accession #M73218), and Mexico [Huang et al., 1992] (GenBank accession #M74506). For the nucleic acid comparison, the 3' end stop codon, TGA, and the poly A tail were omitted. For the peptide sequence comparison, the terminator and downstream amino acids were omitted. The computation for the alignment of sequences using the clustal method with PAM250 residue weight table was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service and the GenBank program [Altschul et al., 1990; Gish and States, 1993]. Primer sequences were determined by the DNASTAR program (DNASTAR Inc., Madison, WI).

Phylogenetic trees were obtained using the GCG Wisconsin package, version 8.0 on a VAX 6620 computer (NIH, National Cancer Institute, Frederick, MD). Nucleotide multiple sequence alignment was created by the PileUp program. The PileUp msf-type output files were used by Distances to create genetic distance matrixes utilizing the Juiken-Contor method. Finally, phylogenetic trees were created by the GrowTree (UPGMA algorithm) from distance matrix output files.

RESULTS

RT-PCR Amplification of HEV

HEV was isolated by affinity capture, and HEV RNA by extraction from a stool specimen of two patients with hepatitis E, one from the Algeria-1980 outbreak (M7) and one from the Chad-1983 outbreak (T3). RT-PCR products spanning portions of ORF3 and ORF2 were produced with primer sets based on the Burma sequence [Tam et al., 1991].

In the ORF3 region a 311 nt fragment was obtained for both isolates. In the ORF2 region, a 634 nt fragment at the 3′end of ORF2 was obtained from the Algerian M7 isolate. The major portion of the ORF2 region of the Chad T3 isolate was amplified as three overlapping PCR products of 591, 540, and 660 nt (Fig. 1). The 3′ end of the ORF2 PCR products from both isolates included the stop codon TGA and the poly-A tail.

Production of the Clones

The amplified products were ligated into a pCRII vector and four transformations were performed yield-

TABLE I. Degree of Nucleic Acid Homology of the Reference Strains and Algerian and Chadian Isolates in the ORF2 and ORF3 Regions of the Genome

	Algerian M7		Chad T3	
Isolates/ strains	3′ end ORF2 (615 nt)	ORF3 overlap (265 nt)	3′ end ORF2 (615 nt)	ORF3 overlap (265 nt)
China A	90 (100)	95 (98)	90 (98)	95 (98)
China B	90 (99)	95 (97)	90 (97)	93 (97)
China C	89 (99)	95 (97)	90 (97)	94 (97)
Myanmar	90 (98)	94 (98)	89 (97)	95 (98)
Burma	90 (100)	95 (98)	89 (98)	96 (98)
Pakistan	89 (99)	95 (98)	90 (97)	94 (98)
India	91 (98)	94 (98)	90 (98)	95 (99)
China D	90 (99)	94 (95)	90 (97)	94 (95)
Mexico	77 (91)	84 (88)	77 (91)	84 (88)

Results are expressed as percent homology of nucleic acid sequences (in parenthesis as percent peptide homology). Algeria/Chad: 615 nt compared in the 3′ end of ORF2: 92 (98); 265 nt compared in the ORF3 region: 95 (98).

ing recombinant plasmids designated as follows: M7A from Algeria M7; T3A, T28, Tnih from Chad T3 (Fig. 1). Three independant clones were isolated for each amplified product.

Analysis of the Consensus Sequences

A consensus sequence was derived from the three clones representing each amplified cDNA fragment for ORF2 or from PCR products for ORF3. In ORF2, consensus sequences derived from clones were confirmed by those directly derived from PCR products suggesting that nucleotide changes observed were not due to amplification errors (data not shown). Each consensus sequence from the ORF3 and ORF2 regions of the genome was compared to the corresponding sequence of each of the 9 reference strains.

In the ORF3 region, the 265 nt consensus sequence (the segment resulting after primer sequences were eliminated from the 311 nt PCR product) from T3O and M7O, obtained for the T3 and M7 HEV isolates, was analyzed. The nucleic acid sequences of the M7O and T3O fragments were 95% homologous to each other. They were 94 to 96% homologous to the Asian strains but only 84% homologous to the Mexican strain (Table I). In the ORF2 region, the 612 nt consensus sequences, T3A and M7A, obtained for the T3 and M7 HEV isolates, were compared to the reference strains. The nucleic acid sequence of the M7A and T3A clones were 92% homologous to each other. They were 88 to 91% homologous to the Asian strains but only 77% homologous to the Mexican strain (Table I). The analysis of the ORF3 and ORF2 regions suggested that at the nucleic acid level, the African isolates formed a separate group, distinct from the Asian strains but more related to this group than to the Mexico strain (Figs. 2 and 3).

The nucleic acid and amino acid sequences of the three portions of ORF2 from the T3 HEV isolate (T3A, T28, and T3nih) were compared to corresponding segments of the reference strains (Table II). No significant differences were found among these three regions of the HEV genome.

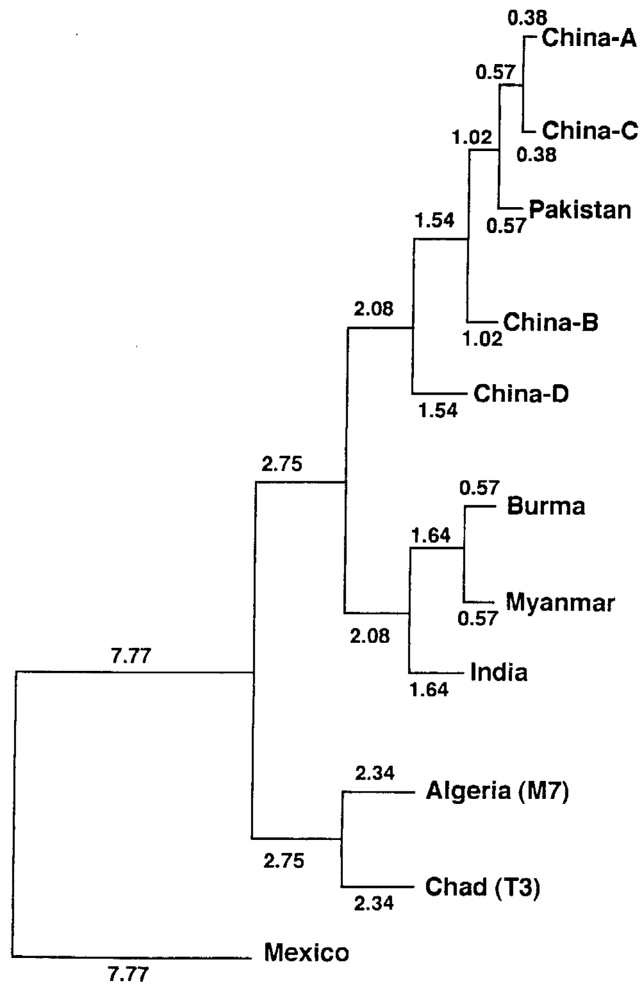

Fig. 2. Phylogenetic tree of the ORF3 region (nt 4996 to nt 5306) for the HEV reference strains and African isolates.

The deduced peptide sequences from the 265 nt fragment in the ORF3 region codes 29 amino acids (aa) from ORF1, 59 aa from ORF3, and 45 aa from ORF2 and must be considered an ORF1,2,3 overlap region (ORF3 overlap). The deduced peptide sequences from Algerian and Chadian isolates were 98% homologous in the ORF3 overlap and ORF2 regions. For both regions, the African peptide sequences were 95 to 100% homologous to the Asian strains and 88% homologous to the Mexican strain. These results suggest that at peptide level, the distinction between the North African isolates, the Asian strains, and the Mexican strain was less significant than at the nucleotide level.

The T3ORF2 peptide, the Mexico ORF2 and the India ORF2 have an isoleucine at position 436 (Fig. 4). This amino acid change results in Chad and India strains being more closely related in the phylogenetic tree to the Mexican strain (data not shown). Remarkably, the Chad T3ORF2 sequence has three unique amino acid changes compared to the reference strains: a Leucine-Glycine substitution in position 3, a Serine-

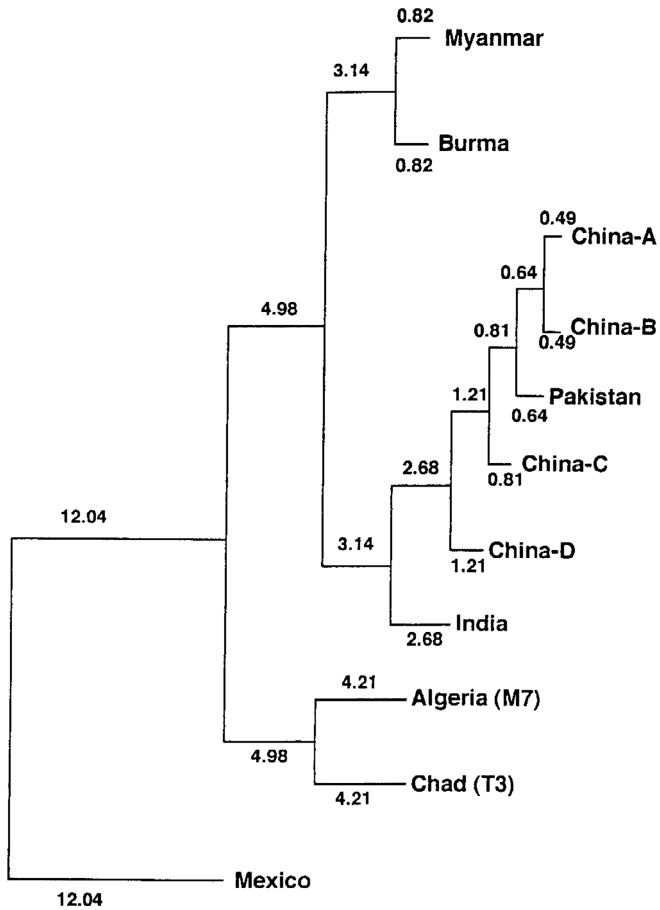

Fig. 3. Phylogenetic tree of the ORF2 region for HEV reference strains and African isolates. The regions compared were from nt 6577 to 7192 for the Algerian isolate and from nt 5550 to 7192 for the Chadian isolate.

TABLE II. Degree of Nucleic Acid Homology of the Reference Strains and Chad T3 Isolate in the ORF2 Region of the HEV Genome

	Chad T3			
Isolates/ strains	3′ end ORF2 (615 nt)	Middle ORF2 (506 nt)	5′ end ORF2 (590 nt)	T3 ORF2 (total) (1,576 nt)
China A	90 (98)	92 (99)	91 (99)	91 (99)
China B	90 (97)	92 (99)	91 (98)	91 (98)
China C	90 (97)	92 (99)	91 (99)	91 (99)
Myanmar	89 (97)	90 (99)	91 (98)	91 (98)
Burma	89 (98)	90 (99)	91 (99)	91 (99)
Pakistan	90 (97)	92 (99)	91 (99)	91 (99)
India	90 (98)	91 (99)	90 (99)	90 (99)
China D	90 (97)	91 (99)	91 (98)	91 (98)
Mexico	77 (91)	78 (92)	78 (94)	78 (94)

Results are expressed as percent homology of nucleic acid sequences (in parenthesis as percent peptide homology).

(Ser)-Threonine(Thr) substitution at position 221, and a Alanine-Valine at position 439. The Ser/Thr amino acid change is semi-conserved (Fig. 4). In addition, the Chad T3A, Algeria M7A, Burma, and Myanmar strains have an amino acid change in position 479 (leucine replaces methionine) which is not found in any of the other strains (Fig. 4).

These results suggest that at the nucleic acid level, the African strains are closely related and form a group separate from the Asian group but more similar to the Asian strains than to the Mexican strain. At the peptide level, the Algerian and Chadian HEV isolates are more similar to the Asian strains than to the Mexican strain.

Nucleotide Sequence Accession Numbers

The nucleotide sequence data of the HEV isolates have been deposited with the EMBL/GenBank database and assigned the following accession numbers: T3 ORF2, from nt 5550 to 7192 (U6221); T3 O, ORF3 overlap (AF001275); M7A (U40046); M7O, ORF3 overlap (AF001276).

DISCUSSION

The ORF3 and ORF2 regions were sequenced directly or after cloning for HEV isolates from hepatitis E outbreaks in Algeria (Mostaganem-1980) and Chad (N'Djamena-1983). In both ORF2 and ORF3 regions analyzed at the nucleotide level, three distinct phylogenetic groups were identified: Asian, African, and Mexican. The Asian group was further divided into two subgroups: Burma/Myanmar subgroup and China/Pakistan subgroup. Interestingly, the India strain was more closely related to either the Chinese or the Burma/Myanmar subgroup, depending on the region of the genome analyzed. The African group was more closely related to the Asian group than to the Mexican strain. The differences observed at the nucleic acid level decreased at the peptide level.

This sequence data may help explain the heterogenous findings in previous studies of the immunoreactivity of synthetic peptides in the ORF2 region.

Serum from patients and primates infected with Burma and Mexico strains cross-reacted with synthetic peptides based on Burma or Mexico sequence in the ORF2 immunodominant regions from amino acid positions 184 to 206, 287 to 302, 427 to 445, and 478 to 525 [Coursaget et al., 1993a; Kaur et al., 1992; Khudyakov et al., 1993, 1994; Yarbough et al., 1991] (Fig. 4). Large expressed ORF2 proteins containing the ORF-2 epitopes showed no significant differences in immunoreactivity against sera from the same or different origins [Tsarev et al., 1993; Yarbough et al., 1991, 1996], except for Egyptian sera which showed a low reactivity [Goldsmith et al., 1992]. However, small synthetic peptides of this region showed variable, reduced [Khudyakov et al., 1993, 1994; Yarbough et al., 1991], or absent [Coursaget et al., 1993a; Kaur et al., 1992] immunoreactivity against sera from different origins. These findings suggest that immunoreactivity is determined by either a different immunodominant linear epitope or by conformational epitopes rather than linear epitopes.

Further, despite the specific amino acid changes unique to the Chad peptide sequence (positions 3, 221, and 439), the amino acid change at position 436 (Ile) in common with the Mexico sequence, or the change at

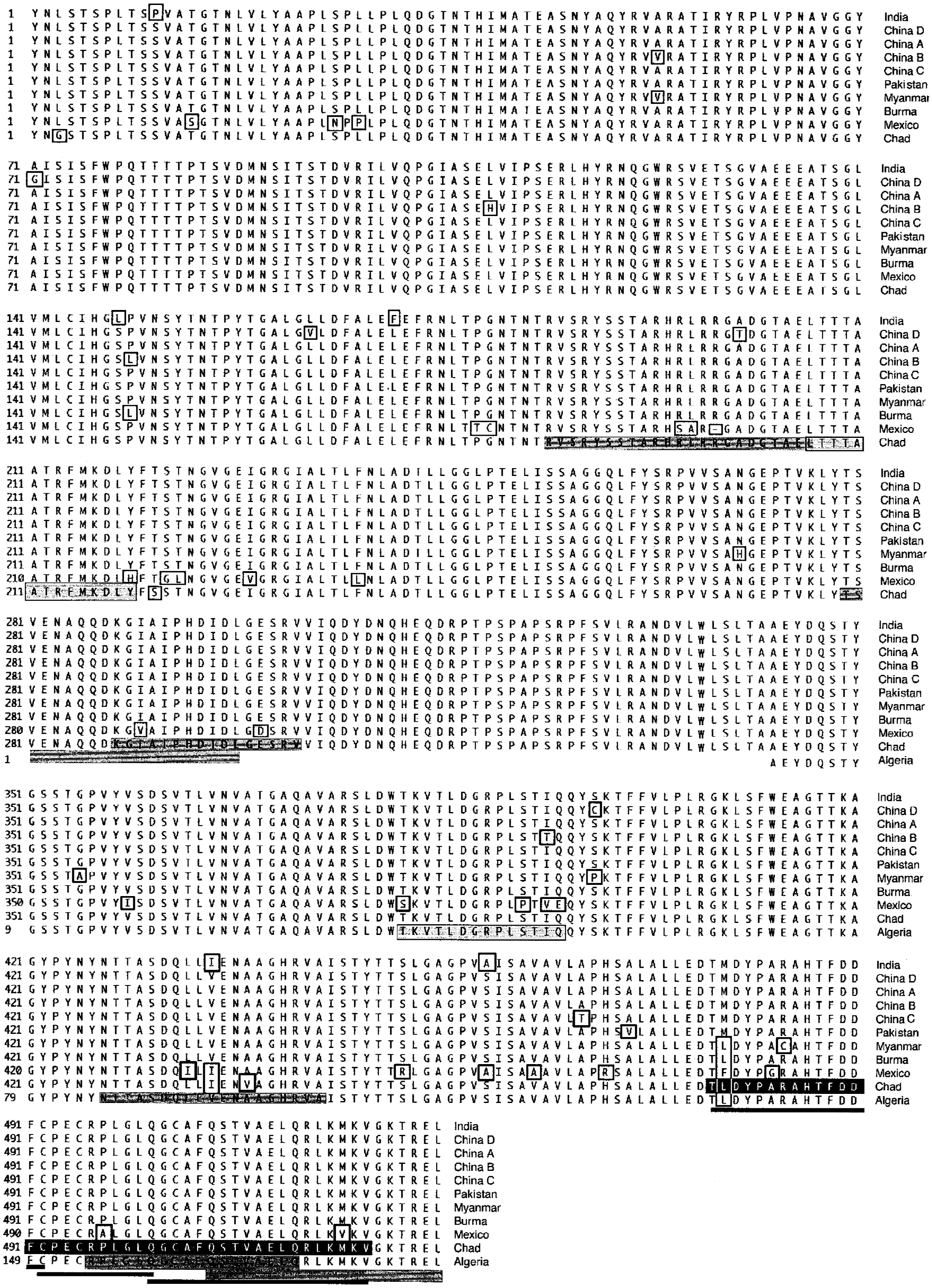

Fig. 4. Alignment report of the peptide sequences in the ORF2 region of the HEV reference strains, Algeria (M7) and Chad (T3) isolates. The sequence of the oligonucleotides in the epitopic regions of ORF2, tested in different studies for immunoreactivity are underlined [Coursaget et al., 1993a]; in clear shadow [Kaur et al., 1992]; in dark shadow [Khudyakov et al., 1993, 1994]; or with black background [Yarbough et al., 1991].

amino acid position 479 (Leu/Met) in common with the Burma and Myanmar sequences, immunologic cross-reactivity was still observed. Natural antigenic cross-reactivity was apparent since sera from a hepatitis E patient in Pakistan was used to capture the Chadian and Algerian HEV for PCR and the fact that sera from the Chad T3 patient reacted in an EIA using a near full-length expressed ORF2 protein based on the Pakistani HEV genome sequence [Tsarev et al., 1993; data not shown]. These facts support the hypothesis that immunoreactivity is based on conformational epitopes.

The changes observed in the peptide sequence of the ORF2 region, for any isolate considered, do not appear to modify the common antigenicity of the HEV capsid. Thus, a vaccine based on one strain may protect against all known HEV strains, including the African strains. However, whether the nucleic acid or amino acid differences among strains influence the transmissibility or infectivity of a strain for a naive individual or an individual previously infected with the same strain or a different strain remains undetermined.

Previous studies divided HEV into two phylogenetic groups, typified by one Mexico strain and a group of strains from Asia. We have shown, based on the nucleic acid sequence, that in the ORF3 and ORF2 portions of the genome studied the Algeria and Chad HEV isolates formed a third distinct group more closely related to Asian strains.

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